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Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists

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ABSTRACT **Recent studies identified a short peptide motif that serves as a docking site for cyclin/cyclin-dependent kinase (cdk) 2 complexes. Peptides containing this motif block the phosphorylation of substrates by cyclin A/cdk2 or cyclin E/cdk2. Here we report that cell membrane-permeable forms of such peptides preferentially induced transformed cells to undergo apoptosis relative to nontransformed cells. Deregulation of E2F family transcription factors is a common event during transformation and was sufficient to sensitize cells to the cyclin/cdk2 inhibitory peptides. These results suggest that deregulation of E2F and inhibition of cdk2 are synthetically lethal and provide a rationale for the development of cdk2 antagonists as antineoplastic agents.**

Certain molecular pathways frequently are altered during human carcinogenesis. Therefore one approach to treating cancer, while minimizing host toxicity, would be to develop drugs that preferentially kill cells in which such pathways are altered.

An example of a molecular pathway that is recurrently altered in cancer involves the retinoblastoma tumor suppressor protein (pRB) (1–3). Many tumors lack a wild-type *RB-1* allele, thus depriving them of pRB. Furthermore, pRB is negatively regulated by cyclin-dependent kinases (cdks). These cdks are, in turn, negatively regulated by certain cdk inhibitors and positively regulated by certain cyclins. These upstream pRB regulators frequently are altered in tumors that retain a wild-type *RB-1* allele. Thus, functional inactivation of pRB, a known inhibitor of cell growth, may be a necessary step in human carcinogenesis.

Members of the E2F cell-cycle regulatory transcription factor family are critical downstream targets of pRB. Binding to pRB converts E2F from a transcriptional activator to a potent transcriptional repressor. Consequently, E2F-responsive genes are activated in cancer cells because of loss of pRB/E2F repressor complexes and liberation of free, transcriptionally active E2F. Of note, at least some E2F family members, including E2F1, are themselves transcribed from E2F-responsive promoters (4–7). Thus, pathological activation of E2F responsive genes can establish a positive feedback loop. Paradoxically, forced activation of E2F-responsive genes, such as through the overproduction of E2F1, can induce both cellular proliferation and cell death (apoptosis) (8–11).

E2F family members bind to DNA as heterodimers with members of the DP family. The DNA-binding capability of some of these heterodimers is negatively regulated by cyclin A/cdk2 (12–15). For example, E2F1, E2F2, and E2F3 each contain a short, collinear cyclin A/cdk2 binding motif that is required for the timely neutralization of E2F DNA-binding capability as cells traverse and prepare to exit S phase (13, 16, 17). Mutation of this motif in E2F1 enhances its ability to induce apoptosis (13). In summary, E2F is negatively regulated by both pRB and cyclin A/cdk2. We therefore reasoned that pharmacologic inhibition of cyclin A/cdk2 might preferentially kill cells in which E2F already was deregulated by virtue of pRB inactivation.

MATERIALS AND METHODS

Cell Culture. U2OS osteosarcoma cells, MDA-MB-435 breast carcinoma cells, HCT116 and SW480 colon carcinoma cells, WI38 human diploid fibroblasts, and the WI38 T-antigen-transformed subline WI38/VA13 were obtained from the American Type Culture Collection. HaCaT human keratinocytes were a gift of Norbert Fusenig, Heidelberg, Germany (18). Rat-1a rat fibroblasts and Rat-1a 1093E2F1 cells have been described (9). SW480 and HCT-116 cells were grown in RPMI medium 1640 supplemented with 10% FCS. All other cells were grown in DMEM supplemented with 10% FCS.

Peptides. Tat peptides were synthesized on an Applied Biosystems ABI 433A peptide synthesizer by using the standard fluorenylmethoxycarbonyl protocol. Amino acid derivatives were purchased from Bachem and Midwest Biotech (Fisher, IN). Reverse-phase HPLC was carried out with a Waters HPLC system on a YMC C18 column by using a linear gradient of CH_3CN/0.1% aqueous trifluoroacetic acid. Purified peptides were analyzed by mass spectrometry. Peptides were fluorescein-labeled on cysteine by using fluorescein-5-maleimide (Pierce) and *N,N*-diisopropylethylamine in dimethylformamide. Penetratin-peptides were purchased from Genosys (The Woodlands, TX).

Kinase Assays. *In vitro* kinase assays were performed as described (16). For Fig. 1*A*, immunoprecipitates were derived from ≈12% of the cells in a nearly confluent 100-mm dish. For Fig. 1*B*, immunoprecipitates were derived from ≈50% of the cells in a nearly confluent 35-mm dish. Kinase reactions were performed in 30-μl reactions containing 200 ng glutathione *S*-transferase (GST)-pRB(792–928).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-((4-Sulfophenyl)-2*H*-Tetrazolium) Chloride (MTS) Assays. Cells (3×10^3)/well were plated in 96-well dishes and allowed to adhere overnight in the presence of 10% FCS. The medium then was discarded, and the cells were washed once with Opti-MEM (GIBCO/BRL). The cells then were incubated in Opti-MEM containing the indicated peptides at various concentrations for 24 hr. For detection of fluorescein-labeled peptides, cells were rinsed once with PBS and visualized by using a fluorescence microscope (Axiovert 135, Zeiss) at ×320

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Abbreviations: cdk, cyclin-dependent kinase; pRB, retinoblastoma tumor suppressor protein; GST, glutathione *S*-transferase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-((4-sulfophenyl)-2*H*-tetrazolium) chloride.

A Commentary on this article begins on page 4221.

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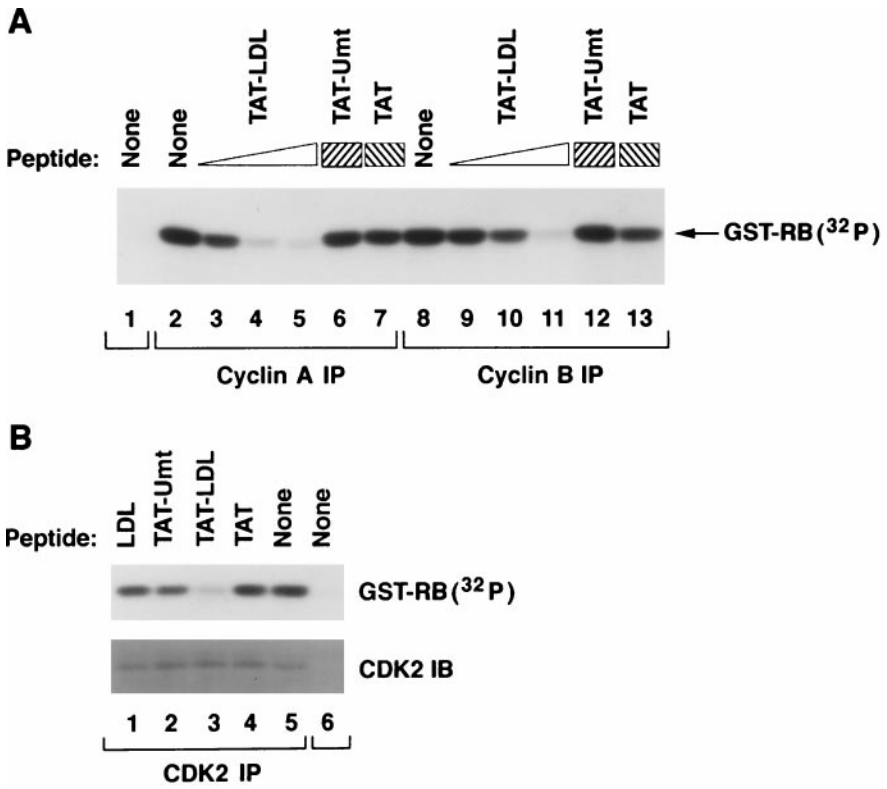

FIG. 1. Peptide inhibition of cyclin/cdk2. (*A*) Phosphorylation of GST-RB *in vitro* by control (lane 1), anti-cyclin A (lanes 2–7), or anti-cyclin B (lanes 8–13) immunoprecipitates in the presence of γ-^{32}P-ATP. Where indicated, TAT-LDL (final concentration 0.1, 1, and 10 μM as indicated by the triangles), TAT-Umt (10 μM), or TAT (10 μM) peptides were added before the addition of substrate. (*B*) Asynchronously growing U2OS osteosarcoma cells were treated with the indicated peptides (50 μM) for 4 hr, lysed, and immunoprecipitated with anti-cdk2 (lanes 1–5) or a control antibody (lane 6). *In vitro* kinase reactions were performed in the presence of γ-^{32}P-ATP using GST-RB as substrate and resolved by SDS/PAGE. Phosphorylation of GST-pRB and recovery of cdk2 were monitored by autoradiography and anti-cdk2 immunoblot analysis, respectively.

magnification. Growth inhibition was measured by using MTS (Promega) assay. One-hundred percent viability refers to the MTS value for mock-treated cells.

Cell-Cycle Analysis. Cell-cycle analysis using fluorescence-activated cell sorting was performed as described with a Becton Dickinson FACSort (9). U2OS cells were treated with the indicated peptide for 6 hr before analysis. Rat-1a cells were treated with the indicated peptide for 24 hr and pulsed with BrdUrd for 1 hr before analysis (9).

RESULTS

We introduced short peptides into cells that can block the interaction of cyclin A/cdk2 with substrates such as E2F1 (16, 19) (Table 1). In the experiments that follow, we used either an E2F1-derived peptide (PVKRRLDL) (LDL) or a consensus peptide (PVKRRLFG) (LFG) based on the cyclin/cdk2 binding motifs in p21-like cdk inhibitors and knowledge of the p27/cyclin A/cdk2 crystal structure (16, 20). Peptides were introduced into cells as chimeras containing N-terminal residues derived from either HIV Tat or the Antennapedia homeodomain protein (penetratin). These sequences direct the efficient uptake of heterologous proteins across cell membranes (21–23).

Table 1. Synthetic peptides

Name	Sequence
Tat	YGRKKRRQRRRG
Tat-LFG	YGRKKRRQRRRG**PVKRRLFG**
Tat-LDL	YGRKKRRQRRRG**PVKRRLDL**
LFG	**PVKRRLFG**
LDL	**PVKRRLDL**
Tat-Smt (scrambled)	YGRKKRRQRRRG**RLDLPKVR**KRS
Tat-Umt (unrelated)	YGRKKRRQRRRG**ETDHQYLAESS**
Pen	RQIKIWFQNRRMKWKK
Pen-LFG	RQIKIWFQNRRMKWKK**PVKRRLFG**
Pen-Umt (unrelated)	RQIKIWFQNRRMKWKK**ETDHQYLAESS**

Previous studies showed that peptides that were similar or identical to the LDL or LFG peptides used here inhibited cyclin A-associated kinase activities but not cyclin B- or cyclin D-associated kinase activity *in vitro* (16, 19). Tat-LDL retained the ability to inhibit cyclin A-associated kinase activity *in vitro* and did so at concentrations approximately 10-fold lower than required inhibition of cyclin B-associated kinase activity (Fig. 1*A*). Cdk2 activity was inhibited in cells within 4 hr of adding Tat-LDL to cell culture media (Fig. 1*B*). This effect was specific and required uptake of the peptide as it was not seen with Tat alone, the LDL peptide alone, or Tat fused to an unrelated peptide (Tat-Umt) (Fig. 1*B*).

The cdk2 inhibitory peptides (Tat-LDL and Tat-LFG) killed U2OS osteosarcoma cells in a dose-dependent fashion (Fig. 2*A*). This effect was specific as these cells were not affected by an analogous peptide in which the cyclin/cdk2 binding motif was altered (Tat-Smt), by Tat fused to an unrelated peptide (Tat-Umt), or by the Tat peptide alone (Tat). Similar results were observed when penetratin (Pen) was used rather than the HIV Tat sequence to deliver a cyclin/cdk2 inhibitory peptide (Fig. 2*B*) as well as in other transformed cell lines (Fig. 2 *C* and *D* and see Fig. 4). In these experiments, cell viability was determined by using a colorimetric assay in which the signal is proportional to the number of functioning mitochondria. U2OS cells treated with the cyclin/cdk2 inhibitory peptides

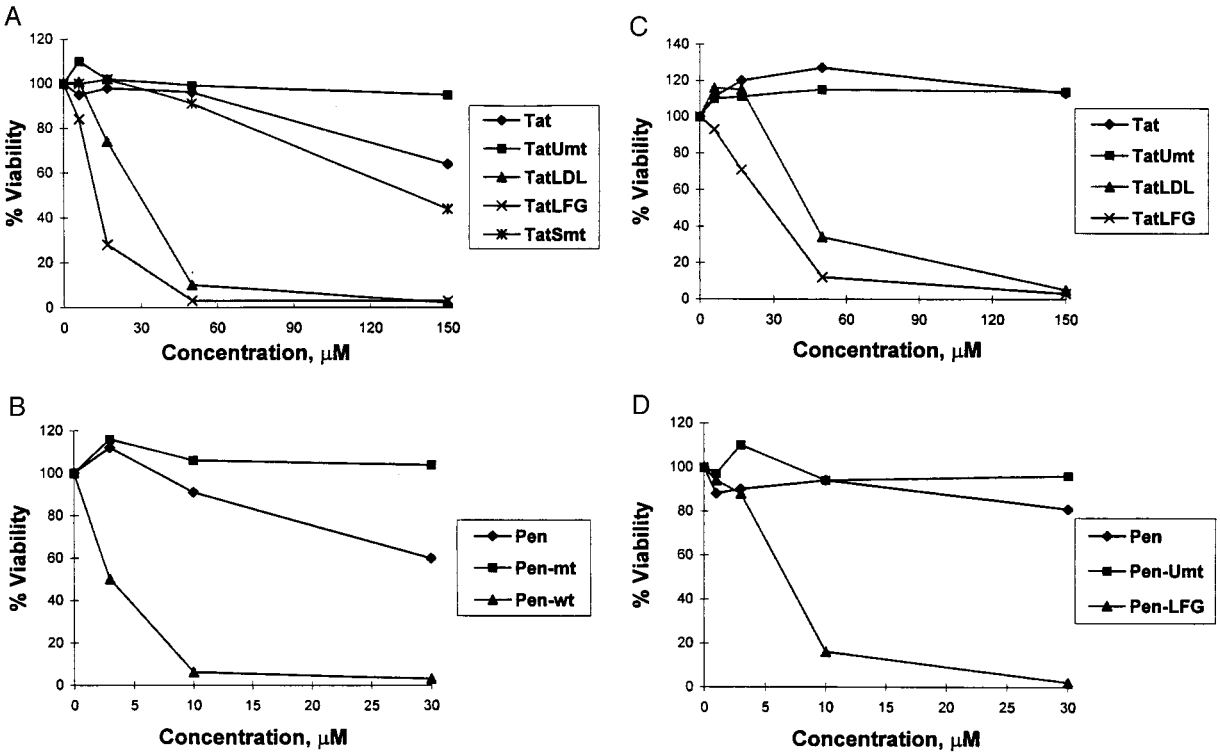

FIG. 2. Cell killing by peptidic cyclin/cdk2 antagonists. U2OS osteosarcoma (*A* and *B*) and MDA-MB-435 breast carcinoma cells (*C* and *D*) were treated with the indicated Tat (*A* and *C*) and penetratin (*B* and *D*) fusion peptides. Cell viability was measured by using an MTS assay.

exhibited nuclear condensation (Fig. 3*A*) and subdiploid DNA content (Fig. 3*B*), suggesting that the observed cell death was the result of apoptosis.

In similar experiments, we found that two nontransformed cell lines (HaCaT keratinocytes and Rat1A fibroblasts) were not affected by the cyclin/cdk2 inhibitory peptides at concentrations that killed transformed cells (Fig. 4*A*). To examine this phenomenon further, we compared WI38 fibroblasts with the T antigen-transformed WI38 subclone VA13. The T antigen-transformed cells were killed by the cyclin/cdk2 inhibitory peptides, whereas the parental WI38 cells were not (Fig. 4*B*). Comparable uptake of the TAT peptides by these two cell lines was confirmed in parallel experiments performed with a fluorescein-conjugated TAT peptide (Fig. 4*C*).

T antigen disrupts the interaction of pRB with E2F. To ask whether deregulation of E2F might account for the differential sensitivity of transformed and nontransformed cells to cdk2 inhibition, we made use of a rat fibroblast cell line stably transfected with an E2F1 cDNA under the control of a zinc-inducible promoter (Rat1a-1093 E2F1). After E2F1 induction these cells were killed by the cyclin/cdk2 inhibitory TAT-LDL peptide but not the control peptide TAT-Umt (Fig. 5*A*). Nuclear morphological changes indicative of apoptosis became apparent within 16 hr of treatment (data not shown). The TAT-LDL peptide also led to an S-phase arrest in cells that were induced to produce E2F1 as shown by double staining with propidium iodide and BrdUrd (Fig. 5*B*). A similar phenotype was described earlier for E2F1 mutants that

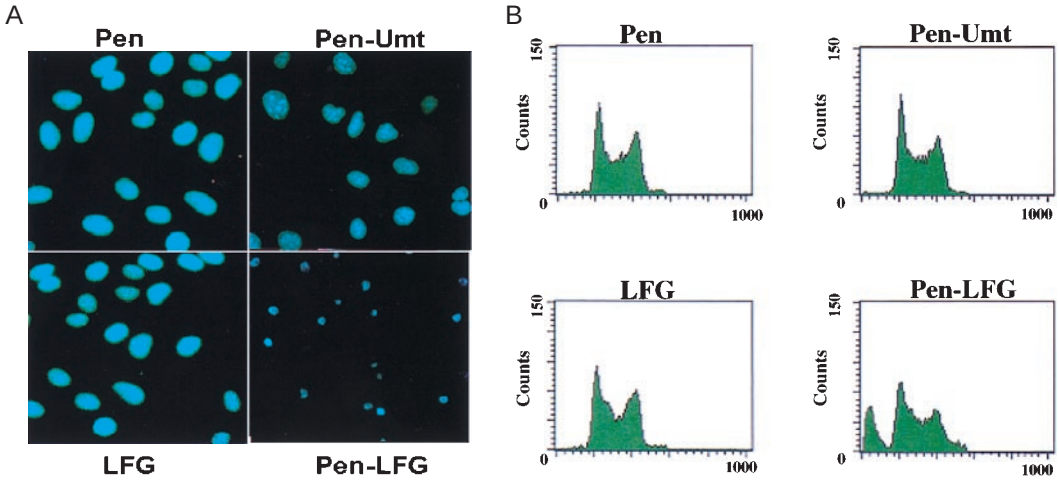

FIG. 3. Induction of apoptosis by peptidic cyclin/cdk2 antagonists. (*A*) Fluorescence microscopy of 4′,6-diamidino-2-phenylindole-stained U2OS cells treated with the indicated peptides. (*B*) DNA content, measured by fluorescence-activated cell sorting after staining with propidium iodide, of U2OS cells treated with indicated peptides for 6 hr at final concentration of 30 μM.

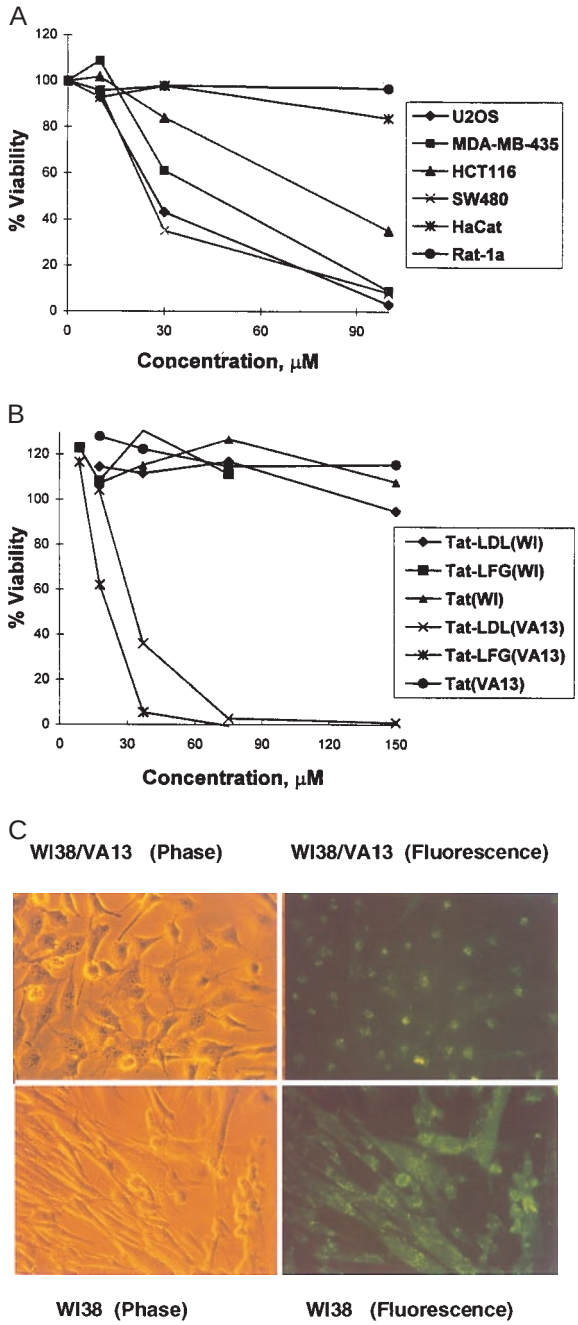

FIG. 4. Preferential killing of transformed cells by peptidic cyclin/cdk2 antagonists. (*A*) The indicated cell lines were treated with the Tat-LDL peptide. Cell viability was measured by using an MTS assay. (*B*) WI38 and WI38/VA13 cells were treated with the indicated peptides, and cell viability was measured by MTS assay. (*C*). Phase-contrast (*Left*) and fluorescence microscopy (*Right*) of WI38/VA13 (*Upper*) and WI38 cells (*Lower*) treated with fluorescein-labeled Tat-LDL peptide.

cannot bind to cyclin A/cdk2 complexes (13). The Tat-Umt peptide alone was inert in these assays (Fig. 5*A* and data not shown). Thus, deregulation of E2F1 and inhibition of cdk2 together lead to cell death.

It was surprising that the cdk2 inhibitory peptides had no gross effect on nontransformed cells in these short-term assays. We therefore serum-starved HaCat cells for 4 days to induce quiescence. The cells then were induced to re-enter the cell cycle in the presence or absence of a cyclin/cdk2 inhibitory peptide. Under these assay conditions, inhibition of cyclin/cdk2 led to a G_1/S block, without evidence of apoptosis, in

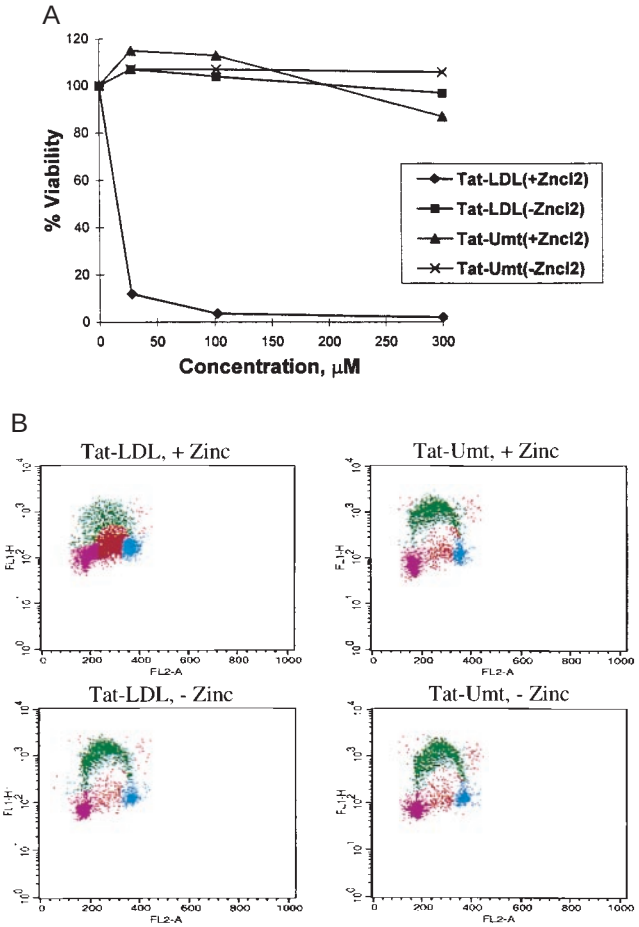

FIG. 5. Peptidic cyclin/cdk2 antagonists kill cells with deregulated E2F. Rat-1a fibroblasts stably transfected with a human E2F1 cDNA under the control of a zinc-inducible promoter (Rat-1a1093E2F1) were treated with the indicated peptides in the presence or absence of zinc. (*A*) Cell viability measured by MTS assay. (*B*) Cell-cycle distribution, measured by fluorescence-activated cell sorting after staining with propidium iodide (*x* axis) and FITC-conjugated anti-BrdUrd (*y* axis) of Rat-1a1093E2F1 cells in the presence (*Upper*) or absence (*Lower*) of zinc. Cells were treated with Tat-LDL (*Left*) or Tat-Umt (*Right*). Cells in G_2/M are shown in blue and cells in S phase undergoing active DNA replication (as measured by BrdUrd incorporation) are shown in green.

keeping with earlier studies in which cdk2 inhibitory antibodies were microinjected into cells (24, 25) (data not shown). In contrast, U20S cells treated similarly underwent apoptosis and exhibited a less-pronounced G_1/S block (data not shown). These results again suggest that immortalized and transformed cells differ qualitatively in their response to cyclin/cdk2 inhibition.

DISCUSSION

We found that short peptides that block the interaction of cyclin A/cdk2 and cyclin E/cdk2 complexes with their substrates selectively and specifically kill transformed cells. The region of cyclin A that binds to the cyclin/cdk2 inhibitory peptides used in our studies is essential for cyclin A function and is relatively conserved among different cyclins (26). This latter observation may account for the ability of these peptides to inhibit cyclin B activity, albeit at higher concentrations than required to inhibit cyclin A activity (Fig. 1*A*). Furthermore, we could not measure the intracellular concentrations of the TAT and penetratin fusion peptides achieved in our studies. Thus, it remains possible that inhibition of cyclins other than cyclin

A and E contributed to the biologic effects induced by these peptides.

Recent advances in molecular oncology have led to the identification of proteins that are quantitatively or qualitatively altered in cancer cells. In many instances these proteins play critical roles with respect to cell growth. Consequently, there is concern that pharmacologic manipulation of these proteins might cause significant host toxicity. It seems likely, however, that some genetic alterations during carcinogenesis are only advantageous, or at least tolerated, in the context of the mutations that temporally preceded them. If true, then normal cells and cancer cells may have markedly different requirements for certain proteins that perform otherwise similar biochemical functions in both cell types. In this regard, it appears that transformed cells and normal cells differ in their requirement for cyclin/cdk2. One plausible explanation, based on our studies, relates to the biochemical and functional interaction of cyclin A/cdk2 with E2F1. In this regard, it is noteworthy that E2F1 can use both p53-dependent and p53-independent pathways to induce apoptosis (9, 27–29). Our findings suggest that cyclin/cdk2 inhibitors might be useful as antineoplastic drugs.

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